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Immunological profile of peripheral blood lymphocytes and monocytes/macrophages in Kawasaki disease

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Summary

Kawasaki disease (KD) is an acute illness of early childhood characterized by prolonged fever, diffuse mucosal inflammation, indurative oedema of the hands and feet, a polymorphous skin rash and nonsuppurative lymphadenopathy. The histopathological findings in KD comprise panvasculitis with endothelial necrosis, and the infiltration of mononuclear cells into small and medium-sized blood vessels. The levels of many proinflammatory cytokines, chemokines and adhesion molecules can be elevated in sera from children with KD at the acute stage. Although many immunological studies on KD involving peripheral blood have been reported, the data obtained remain controversial. This review focuses on the immune response of peripheral blood lymphocytes and monocytes/macrophages during acute KD.

Keywords: Kawasaki disease, peripheral blood, T lymphocytes, monocytes/macrophages, intravenous immnoglobulin

Introduction

Kawasaki disease (KD) is an acute illness of early childhood characterized by prolonged fever, diffuse mucosal inflammation, indurative oedema of the hands and feet, a polymorphous skin rash and nonsuppurative lymphadenopathy [1]. The histopathological findings in KD comprise panvasculitis with endothelial necrosis, and the infiltration of mononuclear cells into small and medium-sized blood vessels [2]. Coronary artery involvement is the most important complication of KD and may cause significant coronary stenosis resulting in ischemic heart disease [3]. Less than 1% of patients with KD may actually die from an aneurysm and/or thrombosis caused by coronary arteritis. Serological testing of patients with this disease reveals nonspecific severe inflammation. The levels of many proinflammatory cytokines, chemokines, and soluble adhesion molecules can be elevated in sera from children with KD at the acute stage [4–9]. In spite of the long history of aetiological investigation in Japan, the cause(s) of KD remains unclear. Recently, two review articles on KD have been reported in 2004 [10,11]. These reviews include the diagnosis, epidemiology, aetiology, pathology, immunopathogenesis and therapy of KD. Although many immunological studies on KD involving peripheral blood have been reported, the data obtained remain controversial. This review focuses on the immune response of peripheral blood lymphocytes and monocytes/macrophages during acute KD, including the effect of intravenous immunoglobulin (IVIG) on peripheral blood monocytes/macrophages.

Numerical changes of peripheral blood lymphocytes and monocytes/macrophages

Table 1 shows the absolute counts of white blood cells, mononuclear cells, monocytes/macrophages and lymphocytes during the acute stage before treatment and during the convalescent stage of KD together with age-matched control subjects [12]. The absolute counts of $CD14^+$ monocytes/macrophages and $CD19^+$ B cells in KD were increased during the acute stage. On the contrary, the absolute counts of $CD4^+$ and $CD8^+$ T cells in KD decreased during the acute stage. These numerical changes of peripheral blood immunocompetent cells are an important finding for investigating the immune response of peripheral blood lymphocytes and monocytes/macrophages during acute KD.

Activation of peripheral blood lymphocytes

The aetiology of KD, particularly the role of staphylococcal and streptococcal superantigens, which have the unique ability to activate a large number of lymphocytes, remains

Table 1. Absolute counts of white blood cells, mononuclear cells, monocytes/macrophages and lymphocytes during the acute stage before treatment and during the convalescent stage of KD and of control subjects.

	Acute stage ($n = 106$)	Convalescent stage ($n = 68$)	Control subjects ($n = 22$)
Mean (SD) day sample taken after onset of fever	5·8 (1·6)	33·9 (19·9)	
White blood cells	15·59 (0·47)†	8·56 (0·29)	8·41 (0·39)
Mononuclear cells	4·58 (0·21)	5·27 (0·20)	4·86 (0·25)
CD14 + monocytes/macrophages	0·52 (0·04)†	0·25 (0·02)	0.18 (0·02)
Lymphocytes	4·06 (0·20)	4·89 (0·19)	4·68 (0·25)
CD4+ T cell	1·81 (0.11)*	2·27 (0·11)	2·33 (0·19)
CD8+ T cell	0·78 (0·04)*	1·14 (0·06)	0·94 (0·06)
CDl9 + B cell	1·23 (0·09)*	1·00 (0·06)	0·94 (0·10)

All results for cell counts are expressed as $\times 10^9/l$ and mean (SEM). *Significant at $P < 0·05$ *versus* control subjects. †Significant at $P < 0·01$ *versus* control subjects.

controversial. Following superantigen activation, T cells with particular T cell receptor β-chain (TCR Vβ) rapidly proliferate [13]. This is followed by T cell Vβ-restricted deletion mediated by Fas-Fas ligand from the peripheral blood, which is one of the processes of apoptosis [14,15]. Thus, the characteristic immunologocal features mediated by superantigens are the response of T cell Vβ expansion and deletion in the peripheral blood of patients exposed to these toxins. Recently, Brogan *et al.* [16] have reported that Class II MHC-positive endothelial cells operate as competent superantigen-presenting cells for CD4 and CD8 lymphocytes, suggesting that activated T cells are temporarily withdrawn from peripheral circulation during acute KD. In addition, the up-regulatinon of MHC Class II expression on lesion endothelial cells has been reported in a patient with fatal KD [17]. This might be related with slight increase of serum interferon γ (IFN-γ) levels in acute KD patients with coronary artery lesions (CAL) reported by us, since IFN-γ increases MHC Class II exression on endothelial cells [18]. At present, conflicting data have been reported regarding expanded T cell populations with particular TCR Vβ gene segments, suggesting either a superantigen- or a conventional antigen-mediated immune response in KD. Although some studies have demonstrated a significant increase or decrease in the percentage of peripheral blood T cells with any particular TCR Vβ family [19–23], the findings have not been confirmed by other investigators [24–27]. We have reported the lack of increases in the serum levels of soluble Fas and Fas ligand during acute KD [28].

The infiltration of activated T cells expressing HLA-DR antigen in biopsy skin lesions and coronary vascular lesions at autopsy has been reported [17,29]. However, it remains uncertain whether peripheral blood T cells are largely activated in acute KD, as some reports have provided evidence of peripheral blood T cell activation [30,31], whereas other reports suggested that there is a low level of activation of peripheral blood T cells during acute KD [32–34]. Recently, we demonstrated a decrease in the number of IFN-γ-producing, but not IL-4-producing, $CD3^+$ T cells in the peripheral blood obtained from KD patients without CAL, suggesting an imbalance of the peripheral blood T cell function at the acute stage [35]. In addition, it has been reported that plasma levels of IL-4 were significantly higher in the acute KD than control children [36]. Our results suggest that some population of peripheral blood T cells, such as IL-4 producing T cells may be activated, while IFN-γ producing T cells (Th1 and Tc1-type $CD3^+$ T cells) develop hypofunction during acute KD. These results further suggest that great caution should be taken in studies on peripheral blood T cell-mediated responses during acute KD.

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, CD152) is a receptor present on T cells that plays a critical role in the down-regulation of antigen-activated immune responses. The expression of CTLA-4 on the T cells depends on cell activation induced by CD28–B7 interaction, which is essential for T cell activation. CTLA-4 is a surface molecule on activated T cells exhibiting sequence homology to CD28 [37]. The essential inhibitory function of CTLA-4 is to maintain the homeostasis of the immune system [38]. We demonstrated that the intracellular T cell expression of CTLA-4 is up-regulated in peripheral blood $CD3^+$, $CD4^+$ and $CD8^+$ T cells in the early part of the acute stage in KD [39]. However, there was a mild increase in intracellular T cells expressing CTLA-4 in KD compared with in Epstein-Barr virus infectious mononucleosis and influenza virus-associated encephalopathy [39,40]. It is an important finding that there is down-regulation of antigen-activated peripheral blood T cell during acute KD, in spite of a mild increase in intracellular CTLA-4 in T cells.

A few studies have been reported on the activation of peripheral blood B cells in KD. The findings included polyclonal B cell activation, an increase in the absolute number of B cells and increased expression of CD23 on B cells [12,41–43]. Recently, it was reported that circulating IgA B cells are reduced in acute KD, while IgA plasma cells infiltrate vascular tissue, including coronary arterial walls, in fatal KD [44,45].

Few studies have been reported concerning natural killer (NK) cells in KD. A significant reduction in the absolute number of circulating $CD16^+$ NK cells was observed during the acute phase of KD [46]. It remains unclear whether the alteration in the number of peripheral blood NK cells is primary or secondary to the pathologic condition of acute KD.

Activation of peripheral blood monocytes/macrophages

The immunological features of monocytes/macrophages observed in patients with KD can be summarized as follows:

- infiltration by the cells is notable in affected tissues in autopsy cases and in skin biopsy specimens from KD patients [17,29];
- the numbers of peripheral blood $CD14^+$ monocytes/macrophages and activated $CD14^+CD23^+$ monocytes/macrophages increase during the acute stage of KD [12];
- there are elevated levels of a variety of serum cytokines, such as tumour necrosis factor α (TNF-α, IL-1 and IL-6, which are considered to be produced by monocytes/macrophages during acute KD [4–6,47];
- peripheral blood mononuclear cells from patients with acute KD spontaneously secrete high levels of TNF-α and IL-1 [48,49];
- increases in the number of peripheral blood $CD14^+$ monocytes/macrophages, serum TNF-α level, IL-6 activity in serum and secretion of IL-1 from mononuclear cells are more evident in KD patients with than in ones without CAL [4,6,12,47,49];
- KD patients with a high level of soluble TNF receptor in their serum seem to be susceptible to CAL [50];
- predominant vascular endothelial growth factor expression and enhanced nitric oxide synthase expression in monocytes have been demonstrated in patients with KD [51,52];
- immunocytochemical and immunoelectron microscopic studies have shown that monocytes partly differentiate into macrophages in the peripheral circulation during the acute stage of KD [53,54].

It has been reported that the $CD14^+CD16^+$(FcγRIII) monocyte/macrophage subpopulation plays a more important role in inflammation [55]. We observed an increase in the number of peripheral blood $CD14^+CD16^+$(FcγRIII) monocytes/macrophages in acute KD, which showed positive correlation with the disease severity [56]. Furthermore, we investigated the activation of nuclear factor kappa B (NF-κB) in peripheral blood $CD14^+$ monocytes/macrophages and $CD3^+$ T cells by means of Western blotting and flow cytometric analyses. NF-κB is a pivotal transcription factor for genes that encode the proinflammatory cytokines, chemokines and adhesion molecules that mediate inflammation [57–59]. As shown in Fig. 1, NF-κB activation was more increased in peripheral blood $CD14^+$ monocytes/macrophages than in $CD3^+$ T cells in KD patients during the acute stage [60]. These findings suggest that the activation of peripheral blood monocytes/macrophages plays an important role during acute KD.

Effect of intravenous immunoglobulin on peripheral blood monocytes/macrophages

IVIG therapy has been reported to be effective in reducing the incidence of CAL in patients with KD [61–63]. There have been a few reports on the effect of IVIG on peripheral blood lymphocytes, neutrophils and cytokines in acute KD, including lymphocyte activation and apoptosis, neutrophil apoptosis and cytokine modulation [42,64–66]. The mechanism of IVIG in immune thrombocytopenic purpura (ITP) has been elucidated. In a murine model of ITP, IVIG increases the expression of inhibitory Fc receptor FcγRIIB on splenic macrophages [67]. However, the mode of action of IVIG in monocytes/macrophages during acute KD is not clearly understood.

IVIG therapy for acute KD seems to decrease the absolute number of circulating $CD14^+$ monocytes/macrophages [68]. We revealed that NF-κB activation in peripheral blood $CD14^+$ monocytes/macrophages is significantly decreased after IVIG therapy during acute KD [60]. Recently, we demonstrated that IVIG inhibits NF-κB activation induced by TNF-α, while it remains unclear whether IVIG acts extracellularly and/or intracellularly in U-937 cells, human monocytic leukaemia cell line. Western blotting of cytoplasmic extracts of U-937 cells revealed that IVIG inhibited the degradation of the IκBα protein, which suppresses NF-κB activation [69]. Further examination is necessary to determine whether or not the data *in vitro* reflects those *in vivo*.

We previously observed an increase in the number of $CD14^+CD16^+$(FcγRIII) monocytes/macrophages in acute KD and a decrease after IVIG therapy, as shown in Table 2 [56]. *In vitro* study of activation of Fc receptor FcγRIII by flow cytometry demonstrated that IVIG decreased the expression of FcγRIII in U-937 cells and peripheral blood $CD14^+$ monocytes/macrophages, and that this phenomenon is transient [69]. On the other hand, IVIG did not affect FcγRIIB expression on the membranes of U-937 cells or peripheral blood $CD14^+$ monocytes/macrophages. More recently, we observed that $CD14^+CD32B^+$(FcγRIIB) monocytes/macrophages were not increased during subacute KD after IVIG therapy [70]. Regarding FcγR expression in peripheral blood monocytes/macrophages during acute KD, the main effect of IVIG therapy may be based on a decrease in $CD14^+CD16^+$ (FcγRIII) monocytes/macrophages, and not an increase in $CD14^+CD32B^+$ (FcγRIIB) monocytes/macrophages.

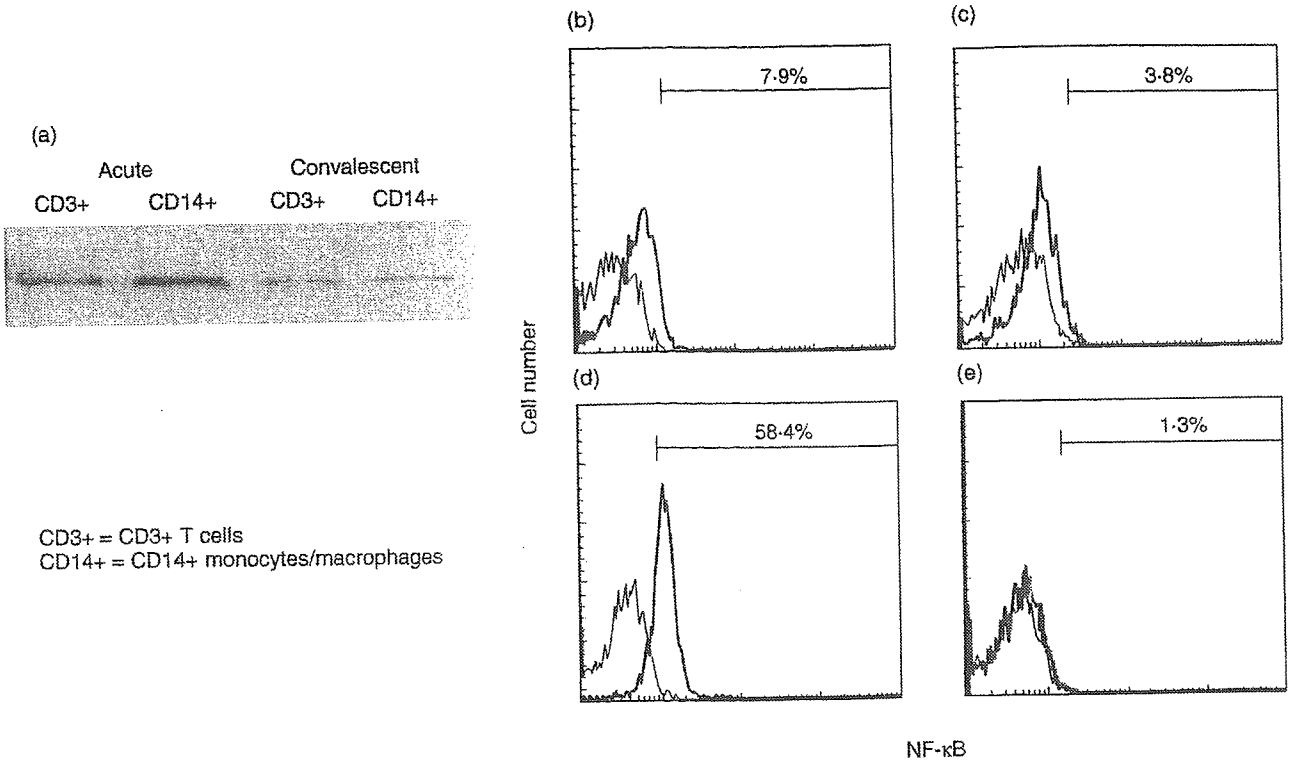

Fig. 1. NF-κB activation in peripheral blood CD3+ T cells and CD14 + monocytes/macrophages of a 2-month-old boy with KD. (a) Nuclear extracts were harvested from CD14+ monocytes/macrophages or CD3+ T cells. The nuclear extracts were used as the sample for Western blotting because activated NF-κB existed in the nucleus. Rabbit polyclonal antibodies against NF-κB-p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the primary antibodies.Western blot analysis demonstrated that intranuclear amount of NF-κB was increased in CD14 + monocytes/macrophages and CD3+ T cells at the acute stage compared with that at the convalescent stage. (b–e) Whole blood was labelled with phycoerythrin-conjugated with anti-CD14 moclonal antibodies and peridinin chlorophyll protein-conjugated anti-CD3 monoclonal antibodies and then permeabilized in 4% paraformaldehyde in phosphate-buffered saline, pH 7·2, containing 0·1% saponin and 10 mm HEPES. The cells were then labelled with a mouse anti-NF-κB (nuclear-localized signal) antibody (IgG3; Boehringer Mannheim, Mannheim, Germany). The mouse anti-NF-κB (nuclear-localized signal) antibody recognizes an epitope overlapping the nuclear location signal of NF-κB-p65 and therefore selectively recognizes the activated form of NF-κB. The cells were then labelled with a FITC-conjugated rat antimouse IgG3 monoclonal antibody (Pharmingen, San Diego, CA, USA). Immunofluorescence was analysed with a FACScan flow cytometer equipped with CellQuest software (Becton-Dickinson Biosciences, San Jose, CA, USA). The percentages of cells with intranuclear NF-κB in CD14+ monocytes/macrophages and CD3+ T cells by flow cytometric analysis are indicated. (b) CD3+ T cells at the acute stage; (c) CD3+ T cells at the convalescent stage; (d) CD14 + monocytes/macrophages at the acute stage; (e) CD14 + monocytes/macrophages at the convalescent stage.

Table 2. CD14+ CD16+ (FcγRIII) monocytes/macrophages in the patients with KD during the acute stage and the convalescent stage, and in control subjects.

	KD ($n = 28$)			
	Acute			Control subjects ($n = 20$)
	Before IVGG	After IVGG	Convalescent	
Mononuclear cells (cells/μl)	5271 ± 2705	5779 ± 2354	5374 ± 2274	5585 ± 1783
$CD14^+CD16^+$monocytes/macrophages (%)	3·6 ± 3·5*	0·6 ± 0·6	0·5 ± 0·3	0·7 ± 0·3
$CD14^+CD16^+$monocytes/macrophages (cells/μl)	155 ± 132*	35 ± 32	25 ± 18	35 ± 18
Percentage of $CD14^+CD16^+$ monocytes/macrophages among $CD14^+$ monocytes/macrophages (%)	21·6 ± 12·5*	6·6 ± 6·8	6·7 ± 3·7	10·1 ± 4·3

Values are expressed as mean ± s.d. * Significant at $P < 0·01$ *versus* convalescent stage and control subjects.

Concluding remarks

Many conflicting data regarding peripheral blood T cell activation during acute KD have been reported. We speculate that these conflicting data might be due to the bipolarity of the peripheral blood T cell function observed in patients with acute KD. There is now ample evidence of a central role of peripheral blood monocytes/macrophages during acute KD, including the observation of an anti-inflammatory action of IVIG on monocytes/macrophages.

Informed consent for participation was obtained from the subjects' parents in our studies. Our studies were approved by the Institutional Review Board of Yamaguchi University Hospital.

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Original article

Low-dose carbamazepine therapy for benign infantile convulsions

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Abstract

Benign infantile convulsions (BIC) are characterized by: (1) onset at up to 2 years of age, (2) normal development, (3) mostly brief, often clustered convulsions, and (4) normal electroencephalography during the interictal stage. BIC follow a favorable course and disappear before 2–3 years of age, although convulsions for which diazepam is ineffective frequently develop. We treated 15 children (3–16 months of age) diagnosed as having BIC, excluding convulsions associated with mild gastroenteritis, with a once-daily dose of 5 mg/kg of carbamazepine until up to 2 or 3 years of age. The serum concentration of carbamazepine was as low as below the effective range in six patients, but the treatment was dramatically effective in all the BIC children. Seizures did not recur in any patients during oral administration of carbamazepine. The treatment was finished in 12 patients at age 2 years, two at age 3 years, and one at 16 months-old. Therefore, we recommend the administration of a once-daily dose of 5 mg/kg of carbamazepine until up to 2 or 3 years of age as a treatment for BIC.

Keywords: Carbamazepine; Benign infantile convulsion; Diazepam

1. Introduction

In 1963, Fukuyama reported benign infantile convulsions (BIC) characterized by onset at up to 2 years of age, generalized tonic-clonic seizures of 2–3 min duration, the absence of underlying diseases, a good prognosis for both psychomotor development and seizures, and normal electroencephalography during the interictal stage [1]. Detailed analysis of electroencephalograms taken during seizures demonstrated that many of the apparent BIC attacks were of secondarily generalized seizures [2]. In addition, the existence of a group of partial epilepsies that develop during childhood and follow a benign course was pointed out, and partial epilepsies with secondarily generalized seizures as the core seizure type representing the majority of what is called BIC have been reported [3]. Furthermore, infants in five families exhibiting bursts of secondarily generalized seizures as an apparently autosomal dominant disorder, designated as benign familial infantile convulsions, have been reported [4]. This disorder disappears before 2–3 years of age. However, seizures frequently recur despite the repeated administration of diazepam as an intravenous injection or suppository. Here, we report that a once-daily dose of 5 mg/kg of oral carbamazepine is effective for BIC. In this study, convulsions associated with mild gastroenteritis were excluded.

2. Patients and methods

2.1. Patients

We explained the study to the parents of the patients, and only studied those whose parents gave their consent. The subjects were 15 patients who visited our hospital, Tsuzumigaura Handicapped Children's Hospital, Ogori Daiichi General Hospital, and Shuto General Hospital between January 1998 and December 2002 (Table 1). They ranged in age from 3 to 16 months, the mean age being 6.9 months, and consisted of six boys and nine girls. On diagnosing BIC, seizures associated with acute encephalitis/encephalopathy, hypoglycemia, electrolyte abnormalities, brain anomalies, cerebral hemorrhage, or mild gastroenteritis were excluded. Of the patients, seven (Patients 4, 5, 6, 10, 11, 13, and 14) were given an intravenous injection or suppository of diazepam before carbamazepine

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Table 1
Clinical data on the 15 patients

Patient number	Age of onset	Sex	Admission	Day of start of therapy from onset	Familial history of convulsive disorders	CT or MRI findings	Follow -up EEG findings
1	6 months	Male	−	2	NP	ND	Normal
2	4 months	Male	+	7	NP	Normal	Normal
3	4 months	Female	+	12	BPEI (mother)	Normal	Normal
4	1 year 4 months	Female	+	1	NP	Normal	Normal
5	5 months	Male	+	1	BPEI (mother and sister)	Normal	Normal
6	8 months	Male	+	2	NP	Normal	Normal
7	3 months	Male	−	9	NP	ND	Normal
8	9 months	Female	+	14	FC (two brothers)	ND	Normal
9	4 months	Female	+	1	FC (father)	Normal	Normal
10	9 months	Female	−	1	NP	ND	Normal
11	7 months	Female	+	8	NP	Normal	Normal
12	9 months	Female	−	17	NP	Normal	Normal
13	7 months	Female	−	8	NP	Normal	Normal
14	3 months	Male	+	20	NP	Normal	Normal
15	9 months	Female	+	31	NP	Normal	Normal

ND, not done; NP, nothing in particular; BPEI, benign partial epilepsy in infancy; FC, febrile convulsions.

administration by the pediatricians who had treated them before us, or before the final diagnosis of BIC. However, seizures recurred in all diazepam-administered patients. The frequency of seizures before the start of carbamazepine administration was 2–30 times (mean, 8.5 times) and the interval between seizures was 5 min to 16 days. The mean interval between seizures for the 24 h before the start of carbamazepine administration for the affected patients was 188 min (range, 5 min–15 h) (Fig. 1). The day of onset of new afebrile seizures was defined as the first day of the disease, and the administration of carbamazepine was started on the 1st–31st (mean, 8.9th) day of the disease.

2.2. Methods

The study protocol was approved by the Ethics Committee of Yamaguchi University Hospital. The oral administration of carbamazepine at a once-daily dose of 5 mg/kg was started immediately after the diagnosis of BIC was established. Since convulsions frequently recurred, carbamazepine was administered as quickly as possible. If the status after convulsions or diazepam-induced sleep did not allow the oral administration of carbamazepine, it was administered through a nasal-gastric tube. Carbamazepine was orally administered at a once-daily dose of 5 mg/kg until up to 2 or 3 years of age.

3. Results

3.1. Acute episodes after the start of administration of carbamazepine

Fourteen of the 15 patients had no recurrence of seizures after the start of administration of carbamazepine. Patient 6 had one seizure at 5 min after the start of carbamazepine administration.

3.2. Follow-up stage

Fig. 2 shows the clinical courses of the 15 patients. Ten of the 15 patients had no seizures during the treatment. In five patients (Patients 1, 2, 3, 9, and 14), seizures recurred after discontinuation of the treatment, and thus the oral administration of carbamazepine was resumed. Finally, seizures did not recur in any patients during the oral administration of carbamazepine.

Twelve of the 15 patients were treated until up to 2 years of age. Two patients were treated until 3 years of age (Patients 1 and 5). In one patient, the treatment was completed at 16 months (Patient 15). In all patients, seizures did not recur after cessation of the treatment. No patients

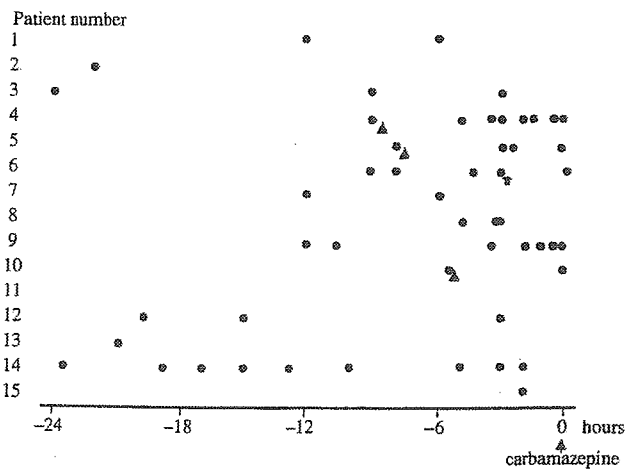

Fig. 1. The frequency of seizures for 24 h before the start of carbamazepine administration to the 15 patients. The time of start of carbamazepine administration is considered to be 0 h. ●, seizure; ▲, suppository administration of diazepam; ▼, intravenous administration of diazepam.

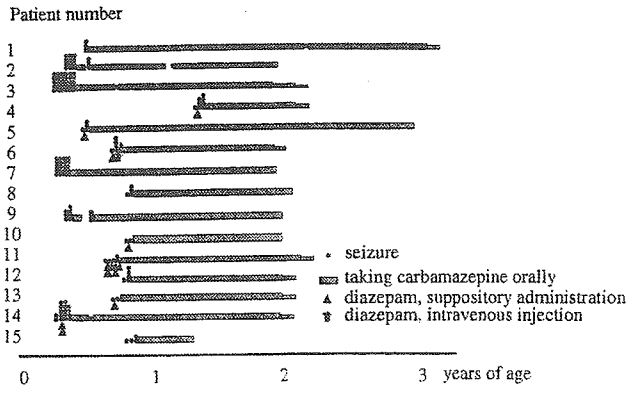

Fig. 2. The clinical courses of the 15 patients.

had any side effects. No patients had neurological sequelae or showed electroencephalographic abnormalities during follow-up.

The serum concentration of carbamazepine was determined in six (Patients 3, 5, 12, 13, 14, and 15) of the 15 patients. The mean serum concentration of carbamazepine at 18.5±4.5 h after oral administration of carbamazepine was 1.8±1.1 μg/ml (range, 0.7–3.85 μg/ml).

4. Discussion

In BIC, clusters of seizures requiring emergency treatment tend to occur. Diazepam is widely used for all types of seizures due to epilepsy, meningitis, and encephalitis; however, this drug is generally ineffective for the treatment of BIC [5,6], as also observed in this study. Previous studies revealed the effectiveness of carbamazepine for BIC [5–7]. However, these studies involved small numbers of patients. In this study, we showed that in acute episodes a once-daily dose of 5 mg/kg of carbamazepine was effective for BIC. At 5 min after the start of carbamazepine treatment, a seizure recurred in Patient 6. Five minutes would be too short for carbamazepine to exhibit an effect on Patient 6.

In the follow-up stage, seizures recurred in five patients who had discontinued the treatment. The range of age at the time of recurrence of seizures in the five patients was 4–26 months. Seizures did not recur in any of 14 patients who had finished the treatment by 2 or 3 years of age. We speculate that treatment with carbamazepine for BIC is necessary by 2 or 3 years of age.

Diazepam has an inhibitory effect by acting mainly on GABA type A-benzodiazepine-picrotoxin receptors, allowing the influx of Cl^- ions from adjacent Cl^- ion channels into neurons [8]. Carbamazepine blocks Na^+ channels and thereby inhibits the influx of Na^+ ions into cells, exerting an anticonvulsant effect [9]. In two families with the combination of familial hemiplegic migraine and benign familial infantile convulsions (BFIC), a mutation in Na^+, K^+-ATPase pump gene ATP1A2 on chromosome 1q23 was observed [10]; and in two families with benign familial neonatal-infantile seizures (BFNIS), a disorder similar to BFIC, one in Na^+ channel gene SCN2A was observed [11]. Thus, judging from the effectiveness of carbamazepine in this study, Na^+ channel abnormalities may cause seizures in BIC. In the present study, the serum concentrations of carbamazepine determined in six of the 15 patients were as low as below the effective concentration range (4–12 μg/ml) [12]. It has been reported that carbamazepine inhibits the binding of [^{3}H] batrachotoxinin A 20-α-benzoate (BTX-B) to Na^+ channel-activating receptors, and that the IC_{50} for BTX-B is 2.4–11.8 mg/kg in rats [13], suggesting that even low concentrations of carbamazepine stabilize Na^+ channels.

Although BIC have a good prognosis, clusters of seizures occurring for several days not only make a patient's family uneasy, but also prolong the hospital stay and thereby increase the hospital expenses. Therefore, we recommend a once-daily dose of 5 mg/kg of carbamazepine for the treatment of BIC until 2–3 years of age. A further control study will be necessary to confirm our idea.

Acknowledgements

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Role of ADAM8 in experimental asthma

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Abstract

A disintegrin and metalloprotease (ADAM) family members, characterized by a metalloprotease and a disintegrin domain, are membrane-anchored glycoproteins involved in proteolysis and cell adhesion. ADAM8 is specifically induced in the experimental murine asthmatic lung. To evaluate novel pathways involved in asthma pathogenesis, using ADAM8 transgenic mice (ATMS2) in a murine model of asthma. Massive cellular infiltrates in peribronchovascular and interstitial lesions were observed in control mice, while in ATMS2 mice there were only occasional. Vascular cell adhesion molecule (VCAM-1) is involved in specific eosinophil adhesions via $\alpha 4\beta 1$ integrin. VCAM-1 shedding was mediated by the ADAM8 metalloprotease. Endothelial cell shedding of VCAM-1 was increased in ATMS2-stimulated human umbilical endothelial cells. ADAM8-mediated shedding of VCAM-1 might be important for the suppression of experimental asthma. Our data suggest that ADAM8 is a useful therapeutic target.

Keywords: Asthma; ADAM8; Vascular cell adhesion molecule (VCAM-1)

1. Introduction

Asthma is a chronic eosinophilic inflammatory disease in which genetic and environmental interactions have an important role. A Th2 inflammatory response to common environmental antigens is a characteristic feature of asthma [1]. The Th2 inflammatory response results in a remodeled airway, smooth muscle hyperplasia, and matrix deposition. Remodeling of the airway predisposes individuals to bronchial hyperresponsiveness. Recently, a disintegrin and metalloprotease domain (ADAM) 33 gene was identified as being significantly associated with asthma and bronchial hyperresponsiveness by using positional cloning to search for disease causing genes [2].

ADAM proteins are expressed in a variety of cells and tissues, including brain, testis, epididymis, ovary, breast, placenta, liver, lung, bone, and muscle. ADAMs are a family of membrane-anchored glycoproteins that are involved in the proteolytic processing of membrane-bound precursors, the modulation of cell–cell and cell–matrix interactions, and issue morphogenesis. ADAM8, also known as CD156, is upregulated in the central nervous system following neurodegeneration and activation of glia cells, astrocytes and microglia, and is suggested to have a role in cell adhesion and cell fusion [3]. ADAM8 has metalloprotease activity in vitro, acting on myeline basic protein, a variety of peptide substrates, and membrane-bound cytokines, growth factors, and receptors [4,5]. ADAM8 is highly expressed in monocytes, neutrophils, and eosinophils [4]. ADAM8 is also called CD156, indicating that it is a leukocyte differentiation antigen that might have an important role in the immune system. Microarray analysis revealed that ADAM8 is highly transactivated in human eosinophils and mouse experimental asthma models [6,7], but its role in allergic reactions is not known. We examined the effect of ADAM8 in an experimental asthma model using transgenic mice (ATMS2) that secrete soluble ADAM8 (sADAM8) from the liver. We report that sADAM8

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significantly inhibited experimental asthma. These results suggest that ADAM8 might have a significant role in allergic disease, especially in cell infiltration.

2. Methods

2.1. Generation and identification of transgenic mice (TG)

The procedure for the generation and identification of ATMS2 was described previously [8]. Age-matched TG and non-TG mice used in these experiments were backcrossed to C57BL/6 (15 generations). All procedures were conducted according to protocols and guidelines approved by the Guidelines for Animal Experimentation of Oita University Faculty of Medicine.

2.2. Sensitization and challenge

Mice were sensitized by intraperitoneal injection of 10 μg of ovalbumin (OVA) and 1 mg of aluminum hydroxide gel in saline on days 0–11. On days 12, 13, and 14, the mice were exposed to 0.1% aerosolized OVA produced by an ultrasonic nebulizer (NE-U12; Omuron, Tokyo, Japan).

2.3. Serum collection

Mice were bled from the heart 14 h after the last challenge. Blood was coagulated at 4 °C followed by centrifugation for 10 min at 7500 rpm. Serum was collected and stored at −80 °C until analysis.

2.4. Collection and measurement of cells in lung

Mice tracheae were cannulated and bronchoalveolar lavage (BAL) was performed three times with 1 ml aliquots of saline. The BAL fluid (BALF) was centrifuged at 4 °C. Supernatants were stored at −40 °C until assay. The total number of BALF cells was counted with a Burker-Turk. Differential cell counts were performed on slides prepared using a Cytospin 3TM centrifuge (Shandon, Pittsburg, PA). A total of 250 cells was counted.

2.5. Histology

Lung obtained from mice before and 14 h after OVA challenge was fixed in 10% formalin, embedded in paraffin, sectioned at 4 μM thickness, and stained with hematoxylin and eosin.

2.6. Cytokines measurement

Mice serum and BALF IL-4, IL-5, and eotaxin concentrations were determined with an enzyme-linked immunoassay (ELISA) using commercially available ELISA kits (Biosource, California). Cytokine concentrations were determined for duplicate wells and reported as the mean ± S.D.

2.7. Endothelial cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were obtained from umbilical cords by collagenase digestion as described by Jaffe [9]. HUVEC were grown in endothelial growth medium (Clonetics, Cambrex Bioscience Walkersville, MD) with 2% fetal bovine serum.

2.8. VCAM-1 shedding assays

Cells were plated at density of 2×10^4 well^{-1} in complete growth medium 24 h before stimulation. Second-passage HUVEC were grown to confluence in 96-well plates. At confluence, cells were washed with serum and corticoid-free medium. Mouse ADAM8 has protease activities for protein of different animal [10]. Cells were cultured with 10% non-TG or ATMS2 serum with or without TNF-α (1000 U/ml, gift from Dainippon Pharmaceutical Co., Osaka, Japan) followed by incubation at 37 °C for 12 h. After stimulation, supernatants were collected and stored at −80 °C until analyzed. Vascular cell adhesion molecule (VCAM-1) concentrations were quantified by an ELISA according to the manufacturer's protocol (Biosource). The levels of soluble VCAM-1 were determined for tetraplicate wells and reported as the mean ± S.D.

2.9. Statistical analysis

The significance of the difference between data was evaluated by the Mann–Whitney *U*-test. A p-value less than 0.05 were considered as statistically significant.

3. Results

3.1. Pulmonary allergic inflammation

There was no change between non-TG and ATMS before the challenge (Fig. 1a and b). In non-TG after the challenge, there were numerous inflammatory cells, including eosinophils, neutrophils, and lymphocytes, infiltrated around the bronchioles; thickened airway epithelium; mucus accumulation in the lumen of bronchioles (Fig. 1c). In ATMS2 after the challenge, there was a reduced number of inflammatory cells; marked reduction in the thickening of airway epithelium, and reduced infiltration of inflammatory cells in the peribronchiolar region (Fig. 1d). These results indicated that sADAM8 inhibit antigen-induced inflammation in the lungs, including the influx of eosinophils.

3.2. BALF analysis

The ATMS2 groups were not significantly different from non-TG with regard to BALF total cell number, percent

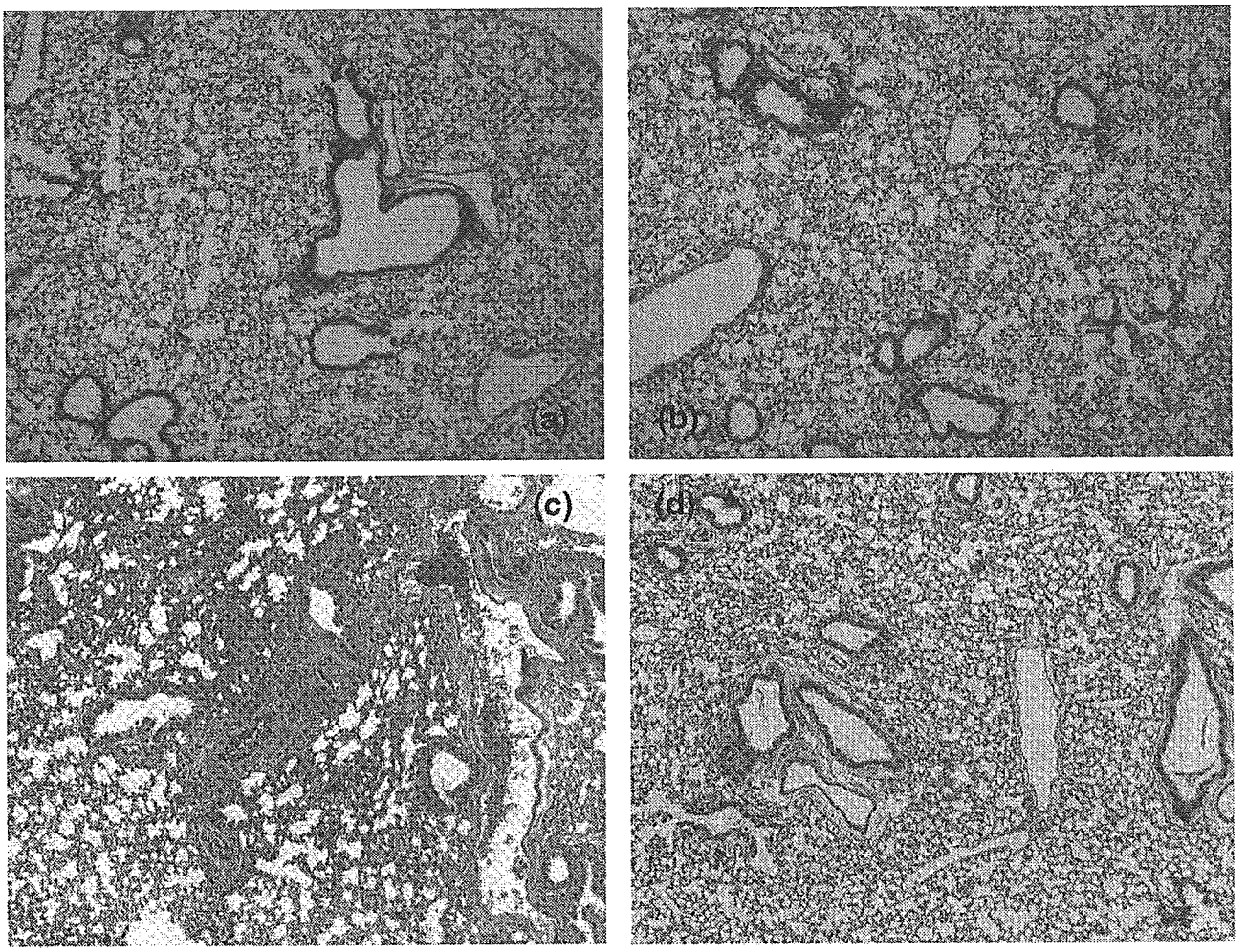

Fig. 1. Representative hematoxylin and eosin-stained lung sections. Sampling was performed before and 14 h after the last challenge in ATMS2 mice and non-TG mice: (a) untreated non-TG; (b) untreated ATMS2; (c) treated non-TG; (d) treated ATMS2.

eosinophils, percent neutrophils, percent lymphocytes, or percent alveolar macrophages, although there were remarkable differences in the histologic examination of lung tissue among the groups (Fig. 2).

3.3. Interleukin (IL)-4, IL-5, and eotaxin levels in serum and BALF

IL-4 levels in BALF were detectable, but there was no significant difference between ATMS2 and non-TG. There was no significant difference of IL-5 in BALF between ATMS-2 and non-TG. Eotaxin levels in BALF after OVA-challenge were significantly elevated in ATMS-2 compared to non-TG ($p < 0.05$; Fig. 3a). These results indicated that the reaction to local OVA stimuli in ATMS2 lung was fundamentally the same as that in non-TG.

Interleukin-4 was not detected in serum before or after OVA-challenge in either ATMS2 or non-TG. IL-5 levels were significantly increased in non-TG after OVA-challenge compared to baseline ($p < 0.05$). IL-5 levels were significantly lower in ATMS2 after OVA-inhalation compared to non-TG ($p < 0.05$). There was a tendency toward reduced levels of serum eotaxin after OVA-inhalation in non-TG and ATMS2. Eotaxin levels were significantly reduced in ATMS2 after OVA-inhalation ($p < 0.05$; Fig. 3b).

3.4. Effect of ATMS2 serum on TNF-α-stimulated VCAM-1 shedding in HUVEC

In a attempt to determine the functional importance of protease properties of sADAM8 on TNF-α-stimulated VCAM-1 shedding in HUVEC, we used ATMS2 serum, which contains high levels of sADAM8 compared to non-TG (8). Incubation with 10% ATMS2 serum for 12 h significantly increased sVCAM-1 shedding from TNF-α-stimulated-HUVEC compared to sVCAM-1 shedding in 10% non-TG serum ($p < 0.05$; Fig. 4). These results indicated that sADAM8 was the protease responsible for VCAM-1 release, including shedding of endogenously expressed VCAM-1 on endothelial cells.

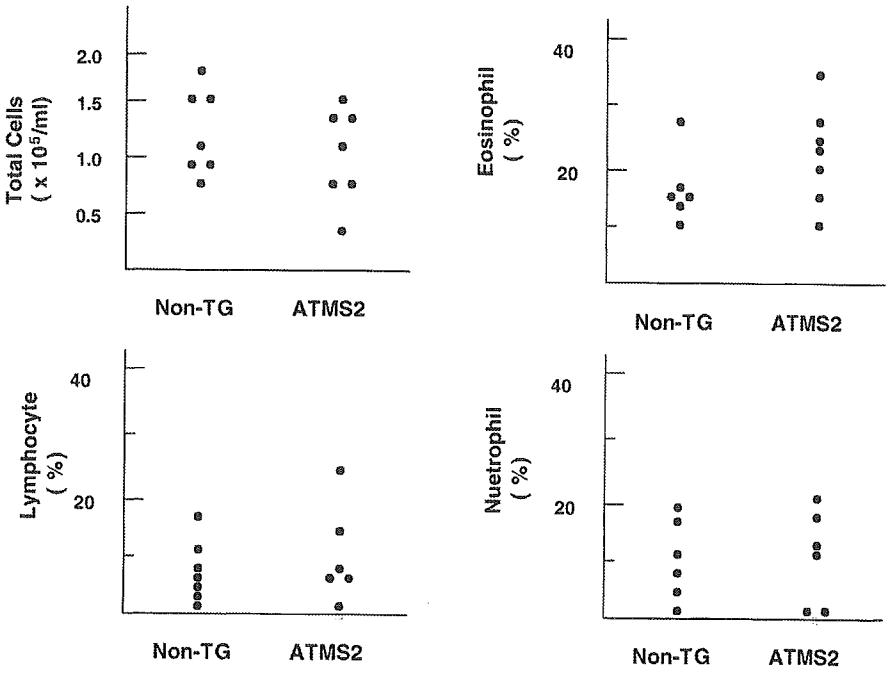

Fig. 2. Analysis of BALF from ATMS2 and non-TG after OVA-challenge of six mice. There were no significant correlations in the number of total cells, percent alveolar macrophages, percent lymphocytes, percent neutrophils, and percent eosinophils.

4. Discussion

We present the first evidence that sADAM8 has a physiologic role in protecting against allergic pulmonary disease in experimental OVA-induced asthma. Histologic examination of lung tissue revealed that ATMS2 had markedly reduced thickening of the airway epithelium and reduced infiltration of inflammatory cells in the peribronchiolar region compared to non-TG. The potential importance of ADAM8 in eosinophil inflammation is highlighted by the recently reported microarray analysis of whole lung from mice in which ADAM8 was one of the mRNAs upregulated after antigen challenge with OVA or Aspergillus fumigatus [11]. Gene-targeted mouse studies revealed that OVA-induced ADAM8 is largely dependent on signal transduction and activation of STAT-6 and the IL-4 receptor α-chain [6]. ADAM8 might have a role in cell migration due to its active metalloprotease domain and also due to potential adhesion properties of its disintegrin. Indeed, the significantly lower level of neutrophil infiltration in ATMS2 following casein-induced peritonitis indicates a role for ADAM8 in leukocyte trafficking [8]. These results suggest that ADAM8 likely contributes to the pathogenesis of allergic lung inflammation, at least in part, by regulating leukocyte migration.

In contract to histologic examination, total cell counts and cell differentiations in BAL were not significantly different between non-TG and ATMS2. While the mechanism responsible for this apparent discrepancy is not clear, potential reasons for the observed finding include increased expression of ADAM8 protein after OVA treatment; there were strong ADAM8 expression levels in inflamed respiratory bronchioles (data not shown). There is no information on cellular interaction via ADAM8 in bronchial cells with leukocyte receptors. The ADAM8 expression in bronchial cells might enable inflammatory cells to inflate into air space.

IL-4 and IL-5, Th2 cytokines, in BALF were equally elevated in ATMS2 and non-TG. Eotaxin levels were significantly higher in ATMS2 compared to non-TG. Local reaction to OVA, therefore, occurred in ATMS2 similar to non-TG, but cellular infiltration was not induced. Therefore, our results suggested that ADAM8 also has an important physiologic role in inflammatory cell infiltration.

Human ADAM8 also naturally occurs in both a membrane and soluble form. ADAM8 might have proteolytic activities to cleave receptors. sADAM8 has metalloprotease activity in vitro and catalyzes myelin basic protein, CHL-1 [5], and CD23, the low affinity IgE receptor [4]. Microarray analysis revealed that ADAM8 is highly upregulated in human eosinophils and in mouse experimental asthma models [6,7].

Eosinophils leave the circulation when adhesion molecules on the cell surface are activated. Subsequent to adhesion, eosinophils migrate between adjacent endothelial cells and into the tissue. Eosinophils migrate across some endothelial cells using adhesion molecules, such as VCAM-1. Johansson et al. reported that ADAM8 is highly expressed in eosinophils and localized to the podosomes upon adherence to VCAM-1 [7]. VCAM-1 mediates cell–cell interactions via binding to its counter receptor, very late antigen-4 (VLA-4), α4β1 integrin, which is involved in recruiting eosinophils to peribronchial lesions in inflamed tissue. VLA-4 is a heterodimeric cell surface molecule found on all hemopoietic mononuclear cells and

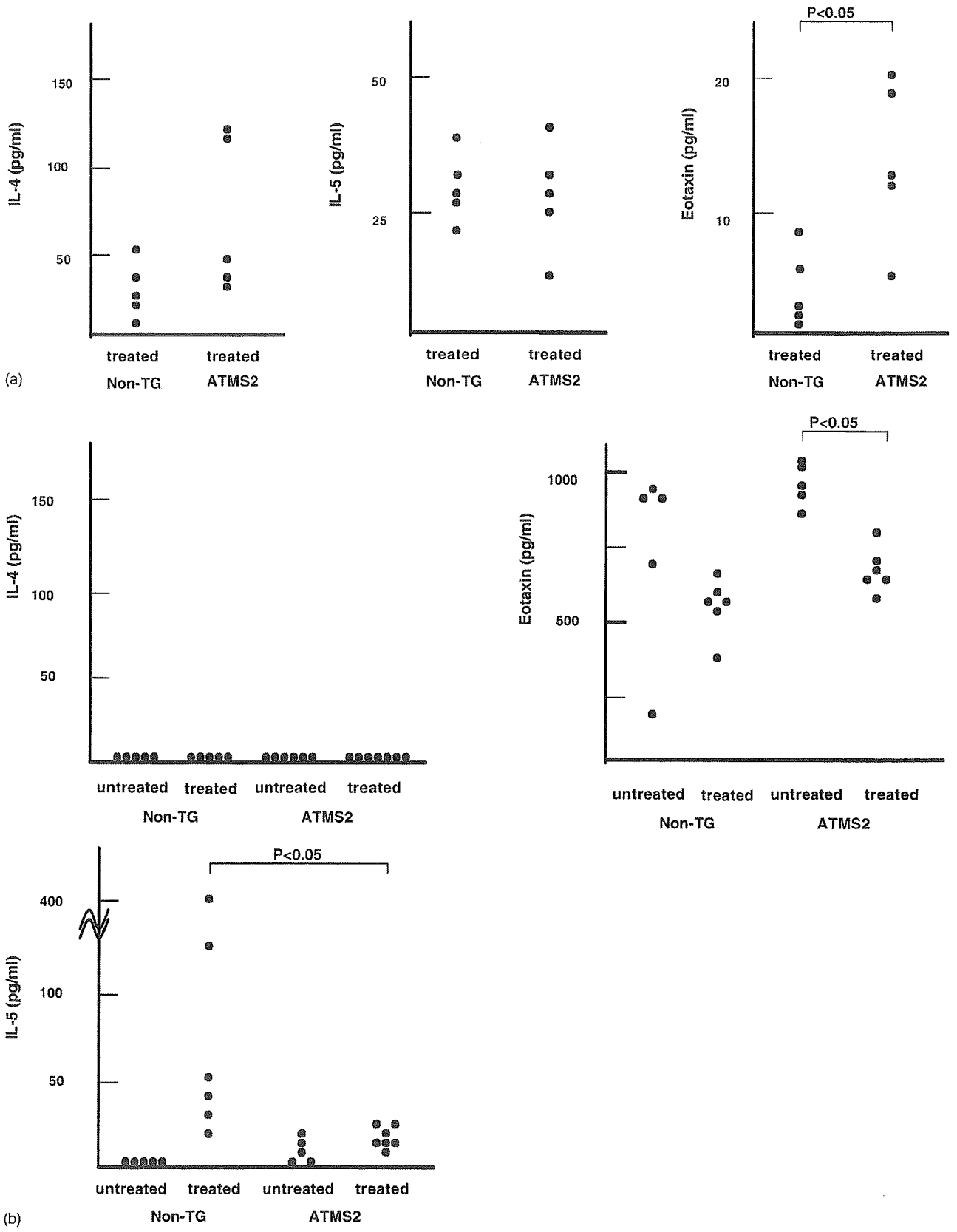

Fig. 3. (a) BALF IL-4, IL-5, and eotaxin levels in ASMS2 and non-TG after OVA-challenge of five mice. There were no significant differences in IL-4 and IL-5 levels between ATMS2 and non-TG. Eotaxin levels in ATMS2 were significantly elevated compared to non-TG. (b) Serum IL-4, IL-5, and eotaxin levels in ATMS2 and non-TG of five mice before and after OVA-challenge. Serum IL-5 levels in ATMS2 after the challenge were significantly lower than those in non-TG. Serum eotaxin levels in ATMS2 after OVA challenge were significantly lower than those before the challenge.

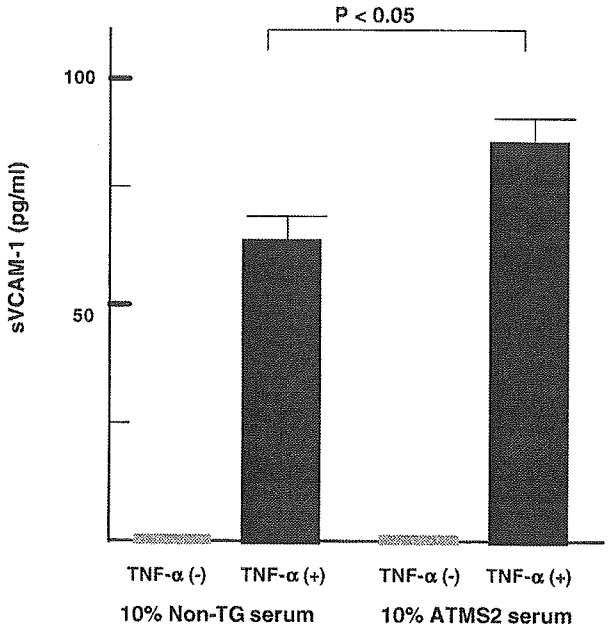

Fig. 4. Evaluation of sVCAM-1 in supernatants from HUVEC in medium containing 10% ATMS2 or non-TG serum are treated with 1000 IU/ml TNF-α as indicated. Values are the mean ± S.E.M. of four experiments. sVCAM-1 levels in ATMS2 were significantly higher than those in non-TG.

eosinophils. On the basis of the cellular distribution and the function of the molecule, VLA-4 antagonists are proposed to be therapeautically effective in allergic disease. Indeed, anti-VLA-4 activity and VLA-4 antagonist inhibit cellular accumulation in experimental asthma [12–14]. VCAM-1 and VLA-4 interaction is necessary for eosinophil migration. Furthermore, adherent eosinophils clear VCAM-1 from the underlying substrate by a metalloprotease-dependent mechanism [15]. TNF-α converting enzyme (TACE, ADAM17) is responsible for one of the proteases induced in the cleavage and shedding of VCAM-1 [15]. TACE mRNA and protein, however, are not regularly expressed in eosinophils [15]. These results indicate that ADAM8 might be the metalloprotease responsible for VCAM-1 substrate degradation by eosinophils.

Shedding of VCAM-1 induced by ADAM8 might help to regulate the adhesive function of VCAM-1 by rapidly decreasing its levels at the cell surface. To determine whether ADAM8 cleaves VCAM-1, HUVEC activated by TNF-α were incubated with ATMS2 or non-TG serum and VCAM-1 cleavage was monitored by ELISA. Soluble VCAM-1 was significantly increased in ATMS2 compared to non-TG. Although the in vivo significance of VCAM-1 cleavage and shedding is not known, the high levels of sVCAM-1 in ATMS2 serum in vitro suggest a physiologic roles. Thus, cleavage of VCAM-1 under pathologic conditions could result in less inflammation of eosinophilic lung disease.

These results demonstrate that sADAM8 might have a suppressive role in leukocyte trafficking by VCAM-1 clearance from endothelial cells. Our data indicate that ADAM8 can be added to the list of proteases that mediate the cleavage and shedding of VCAM-1. On the other hand, the magnitude of the VCAM-1 shedding by plasma from ATMS2 is not high. Therefore, it is expected that another mechanisms would involve in this experiments. It is known that disintegrin domain of ADAM have effect on cell adhesion. The ADAM8 disintegrin domain might interact with different integrins and be involved in neuronal cell adhesion [16]. The ADAM8 disintegrin domain has a protective effect on experimental encephalomyelitis, an animal model for multiple sclerosis [17].

The disintegrin domain of ADAM8 also might play an important role in signal transduction and adhesion. Kenneth et al. reported that anti-ADAM8 disintegrin monoclonal antibody increased neutrophil adhesion to HUVEC [18]. The sADAM8 disintegrin domain in ATMS2 likely interferes with cell surface ADAM8, resulting in cell-to-cell contact inhibition. There is no information, however, on cellular interaction via ADAM8 disintegrin with integrins or endothelial cell receptors. Our studies did not reveal whether the disintegrin activity had an important role in OVA-induced experimental asthma. Further studies are required to evaluate the role of the disintegrin domain in allergic disease.

Cleavage of VCAM-1 by ADAM8 might not induce suppression of allergic lung disease alone, but we conclude that ADAM8 could be involved in the pathogenesis of asthma and is thus a potential target for therapeutic intervention in allergy and inflammation. However, further studies are needed to elucidate the relevance of these observations in human.

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OBSERVATION

Postinfectious Myeloradiculoneuropathy With Cranial Nerve Involvements Associated With Human Herpesvirus 7 Infection

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Background: Infection with human herpesvirus 7 (HHV-7) generally results in a febrile illness with accompanying exanthema subitum.

Objectives: To ascertain and describe the role of HHV-7 in a case of acute myeloradiculoneuropathy.

Patient: A previously healthy young man with complaints of motor weakness, dysphasia, and nasal voice.

Methods: Serological examinations were performed with the patient's serum. We also examined virus genome DNA in cerebrospinal fluid by regular and real-time polymerase chain reaction. Moreover, we checked the antiganglioside antibody level in the patient's serum samples by the immunoblot analysis.

Results: Serological studies revealed significant change in titers of antibodies against cytomegalovirus, Epstein-Barr virus, and HHV-7, but only HHV-7 genome was detected in the cerebrospinal fluid, with its disappearance after therapy. No antiganglioside antibody was detected in the patient's serum.

Conclusion: The unique clinical picture of the present patient might be closely related to the reactivation of HHV-7 in the nervous system.

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GUILLAIN-BARRÉ SYNDROME (GBS) has been recognized as a postinfectious autoimmune disorder against the peripheral nervous system, characterized by acute muscle weakness and areflexia.[1] Many GBS cases have antiglycosphingolipid antibodies such as GM1 ganglioside in patients with *Campylobacter jejuni* infection[2] and GM2 ganglioside, which shares common epitopes between the infectious agents and peripheral nerves, in patients with cytomegalovirus (CMV) infection.[3]

Previous studies have shown that one of the most common classes of viral infection that precedes GBS is the family of herpesviruses. Of GBS cases with respiratory insufficiency and cranial nerve involvement, roughly 10% to 13% and 8% to 10% demonstrate serological evidence of recent exposure to CMV and Epstein-Barr virus, respectively.[4] Another group of the herpesvirus family includes human herpesvirus (HHV) 6 and HHV-7. Primary infections with either HHV-6 or HHV-7 generally occur in children and are characterized by exanthema subitum and febrile illness.[5,6] Human herpesvirus 6 is recognized as an opportunistic pathogen that causes limbic encephalitis in persons infected with human immunodeficiency virus.[7] Human herpesvirus 7 has recently been described as a cause of encephalitis and myelitis in immunologically competent adults.[8,9]

We report a case of acute myeloradiculoneuropathy mimicking GBS, with genetic evidence documenting the presence of HHV-7 in the cerebrospinal fluid (CSF).

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REPORT OF A CASE

A 26-year-old man was admitted to the hospital with a 2-day history of progressive motor weakness, tingling in the extremities, dysphasia, and nasal voice. He had preceding flu-like symptoms 2 weeks before admission. Initial neurological examination revealed moderate motor weakness in the extremities (score of 3 to 4 of 5 on the Medical Research Council scale), with mild hyperreflexia except for the absence of an Achilles tendon reflex. The plantar response was initially flexor and then temporarily extensor. There was evidence of cranial nerve involvement including the facial, glossopharyngeal, and hypoglossal nerves, and autonomic dysfunctions were manifested as a heart conduction block. Examination results of CSF samples taken at admission were normal, but successive examinations demonstrated an increase in protein (89 mg/dL

Table. Changes in Serum Virus Titer and Virus DNA in Cerebrospinal Fluid Before and After Treatment

	Before Treatment (Day 1)	After Treatment (Day 20)	Normal Range
Serum virus titer			
Mumps virus	<4	4	<4*
Cytomegalovirus	<4	16	<4*
Coxsackie B1 virus	<4	<4	<4*
Coxsackie B2 virus	<4	<4	<4*
Coxsackie B3 virus	<4	<4	<4*
Coxsackie B4 virus	<4	<4	<4*
Herpes simplex virus	<4	<4	<4*
Varicella-zoster virus	<4	<4	<4*
Epstein-Barr virus			
EBNA IgM†	-	-	-
EBNA IgG†	-	+	-
Human herpesvirus 6 (IgG)	<4	<4	<4‡
Human herpesvirus 7 (IgG)	16	64	<4‡
Virus DNA amplification by PCR in CSF, copies/mL§			
Human herpesvirus 6	0	0	0
Human herpesvirus 7	2800	0	0
Cytomegalovirus	0	0	0
Epstein-Barr virus	0	0	0

Abbreviations: CSF, cerebrospinal fluid; EBNA, Epstein-Barr nuclear antigen; PCR, polymerase chain reaction; + sign, positive for; - sign, negative for.
*The values were obtained with the complement fixation test.
†The values were obtained with the enzyme-linked immunosorbent assay method.
‡The values were obtained with immunofluorescence method.
§The PCR amplification was performed on the supernatant of CSF samples after centrifugation.

[normal level <40 mg/dL]) and IgG (23 mg/dL [normal level <4 mg/dL]) levels that was accompanied by a modest pleocytosis (8 cells/μL [normal level <5 cells/μL]) by day 20. Laboratory evaluation results for evidence of immunological compromise were negative. A nerve conduction study performed on day 2 and day 25 documented a decrease in compound muscle action potential amplitudes with a reduction of the F-wave frequency. Motor nerve conduction velocities and distal latencies were preserved. There were no temporal dispersions or conduction blocks. Sensory nerve conduction study results were normal. Auditory brainstem response as well as magnetic resonance imaging results of the brain and the spinal cord with gadolinium enhancement appeared normal and, therefore, did not support a diagnosis of brainstem encephalitis. We tentatively diagnosed the patient as having acute myeloradiculoneuropathy, and we treated him with a high dosage of intravenous immunoglobulin (400 mg/kg per day) for 5 days. After treatment, complete recovery of cranial nerve dysfunction was noted within a week, and motor weakness recovered gradually, with pronounced hyperreflexia in the extremities without pathological reflexes. Eight months after the onset of neurological symptoms, his muscle strength returned to subnormal levels (score of 4 to 5 of 5 on the Medical Research Council scale).

VIROLOGICAL AND SEROLOGICAL STUDIES

We performed serological testing and found a significant change in the serum titers of antibody against CMV and Epstein-Barr virus (**Table**) during the 2-week interval without evidence of a recent *C jejuni* infection, although DNA of neither virus was detected in the CSF by polymerase chain reaction, suggesting cross-reacting (heterologous) antibody responses to CMV and Epstein-Barr virus. We further investigated HHV-6 and HHV-7 DNA in the CSF on day 1 and day 20 (after treatment) by real-time polymerase chain reaction,[3] and we found a significant decrease in the amount of HHV-7 DNA (2800 copies/mL to 0 copies/mL), although no HHV-6 or HHV-7 genomes were detected in the serum sample. Fluorescent antibody testing of serum samples on day 1 and day 20 demonstrated an increase in anti–HHV-7 titers from 1:16 to 1:64 (Table).

ANTIGANGLIOSIDE ANTIBODY

To evaluate the patient's serum for the presence of antiganglioside antibodies, mixtures of gangliosides (GM1, GM2, GM3, GD1a, GD1b, GT1b, GQ1b, and asialo GM1) processed by thin-layer chromatography (using a solvent of chloroform, methanol, and 0.02% calcium chloride in a 55:45:10 vol/vol/vol ratio) were blotted onto a polyvinylidene difluoride membrane by an electrothermal blotter (ATTO Co Ltd, Tokyo, Japan). This polyvinylidene difluoride membrane was probed using patient sera taken on day 1 and day 20 (×1000 dilution) in blocking buffer (2% nonfat milk in the wash buffer, which was phosphate-buffered saline containing 0.5% Nonidet P-40 [Nakarai Tesque Inc, Kyoto, Japan]). After treatment with the second antibody, a positive band was sought using an enhanced chemiluminescence reagent (New England Nuclear, Boston, Mass). A band was present in the positive control (anti-GM1–antibody positive), but no band was detected using the patient's serum samples (**Figure**).

COMMENT

Human herpesvirus 6 can be silently harbored in the human brain following primary infection.[11] Detection of vi-